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April 14, 2000	Mary Rutkowski	Mary Rutkowski
(Date)	(PrintedName)	(Signature)

Docket No. MOG 57688/UST

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

APPLICATION OF: MAARTEN HENDRICK STUIVER ET AL.

SERIAL NUMBER: 09/469,812 **GROUP ART UNIT: 1649**

FILED: December 22, 1999 **EXAMINER: Not Assigned**

FOR: Plasmids For Plant Transformation and Method For Using Same

Box Missing Parts
Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Transmittal of Certified Copy of Priority Application

Attached please find the certified copy of the foreign application from which priority is claimed for this case:

Country: EPO
Application number: 97201990.5
Filing date: June 30, 1997

Respectfully submitted,
ZENECA Ag Products Inc.

Dated: April 14, 2000

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Europäisches Patentamt	European Patent Office	Office européen des brevets

Bescheinigung	Certificate	Attestation
Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.	The attached documents are exact copies of the European patent application described on the following page, as originally filed.	Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr.	Patent application No.	Demande de brevet n°
	97201990.5	

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

M. B. BIJLING

DEN HAAG, DEN
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LA HAYE, LE

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.:
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Anmelder:
Applicant(s):
Demandeur(s):
MOGEN INTERNATIONAL N.V.
NL-2333 CB Leiden
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Plasmids for plant transformation and method for using same

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Bemerkungen:
Remarks: The original title of the invention reads as follows: "Novel plasmids for plant transformation and method for using same"
Remarques:

NOVEL PLASMIDS FOR PLANT TRANSFORMATION AND METHOD FOR USING SAME

FIELD OF THE INVENTION

This invention relates to the *Agrobacterium* mediated plant transformation, especially to transformation of plants with T-DNA, where accidental transfer of non-T-DNA vector sequences is prohibited.

BACKGROUND ART

Transformation of plants by using *Agrobacterium* and the Ti or Ri plasmids present in wild-type *Agrobacterium* bacteria has been known since 1983 (e.g. in EP 0 116 718 and EP 0 120 516). This transformation procedure generally consists of infection of plants with non-tumorigenic *Agrobacterium* strains which have been provided with a heterologous gene. This heterologous gene is located on a plasmid in a piece of so-called T-DNA, which is the DNA located between two imperfect direct repeats of about 24 basepairs length, the T-DNA borders. Transfer of the heterologous gene into the plant takes place in a process wherein the also on the plasmid located *vir*-genes are activated through phenolic compounds by incubation of *Agrobacterium* with plant cells. These vir-proteins (D1 and D2) cause nicking of the border repeats at a precise site, whereby the T-DNA is cut at the T-DNA borders from the plasmid and inserted into the plant genome.

The right border region seems to be the most essential in T-DNA transfer: Ti-plasmids with the T-DNA right border region deleted are avirulent (Holsters, M. *et al.*, Plasmid 3, 212-230, 1980). Deletion of the left border region has no effect on virulence (Joos, H. *et al.*, Cell 32, 1057-1067, 1983).

The necessity for the T-DNA borders to be present remains when the transformation is done using a binary vector system, in which the T-DNA is located on a separate independent replicon, the binary vector.

After transformation the T-DNA is present in the genomes of the host plants as single units or in multiple, tandemly arrayed copies. However, truncated T-DNA regions are also frequently observed (Deroles S.C. and Gardner, R.C., Plant Mol. Biol. 11, 365-377, 1988). More recently, there is information that also DNA beyond the borders is integrated into the genome of the host plants. Such is reported to be the case in 20 to 30% of the transgenic plants (Martineau, B. *et al.*, The Plant Cell 6, 1032-1033, 1994). However, very recently, a report in the literature conveyed that approximately 75% of tobacco transformants contained vector 'backbone' sequences (Kononov, M.E. *et al.*, The Plant J. 11(5), 945-957, 1997).

Another phenomenon that is sometimes occurring is that the transfer of T-DNA starts on the left border, which can also act as a (weak) starting point. Then the amount of DNA which is 'read-through' can be substantial: it is found that sometimes the transfer, which starts at the left border, reads through the right border and ends again at the left border, resulting in transfer of the complete binary vector (van der Graaff, E. *et al.*, Plant Mol. Biol. 31, 677-681, 1996).

Since it will be the aim of the plant geneticist to transfer only the DNA that is present in the T-DNA a prohibition of both read-through mechanisms would be welcomed. Furthermore, registration authorities, dealing with requests for (market) registration of transgenic plants and/or transgenic food, also are of the opinion that contamination of transgenic plants with vector DNA should be avoided as much as possible.

SUMMARY OF THE INVENTION

The invention comprises a vector for plant transformation comprising a T-DNA with flanking T-DNA borders, characterized in that the vector further comprises a nucleic acid sequence which prevents the development of plant transformants having more vector sequences than the T-DNA sequence.

This sequence which prevents the development of transformants having more vector sequences than the T-DNA sequence is a gene coding for a toxic compound, preferably selected from the group of RNAse, DNAse,

phytotoxins, diphteria toxin, proteases and antisense housekeeping genes, such as ATP synthase, cytochrome c, pyruvate kinase, aminoacyl transferase, or phosphate, di-, tricarboxylkate and 2-oxo-glutarate translocators.

Another embodiment of said vector is when the sequence which prevents the development of transformants with vector sequences outside the T-DNA sequence by comprising a sequence which binds DNA-binding proteins, or which is high in its G+C-content.

Also part of the invention is a method for obtaining transgenic plants which do not contain vector sequences outside the T-DNA by transforming plants with a vector according to the invention.

Next, hosts containing such a vector, like bacteria, preferably a member of the Agrobacteriaceae, more preferably *Agrobacterium* or *Rhizobacterium*, most preferably *Agrobacterium tumefaciens* form also part of the invention.

Furthermore, a method for the transformation of plants characterized in that a vector according to the invention is used is comprised in the inventionm

DESCRIPTION OF THE FIGURES

Figure 1: Structure of pMOG 1256

Figure 2: Structure of pMOG 1257

DETAILED DESCRIPTION OF THE INVENTION

Basically all known plasmids and vectors which are used for plant transformation can be adapted to a vector according to the invention or a vector usable in a method according to the invention. Examples of such plasmids are pBin19 (Bevan, M., Nucl. Acids Res. 12, 8711-8721, 1984), pMOG101 (WO 93/10251), pMOG800 (WO 95/05467), pMON128 (EP 0 131 623), GV2260 (Deblaere et al. 1985, Nucl. Acids Res. 13, 4777-4788), etcetera and all plasmids derived from them.

The key characteristics of such plasmids are that they contain T-DNA flanked by T-DNA border sequences. Furthermore, they must be suited to replicate in *Agrobacterium*, thus they should contain an origin of replication (ori) sequence suitable for use in *Agrobacterium*. Although the *Agrobacterium*-strains which are most

frequently used are *A. tumefaciens*, also *A. rhizogenes* is suitable for transformation of plants. Here, the transformatable DNA is located on the Ri-plasmid and this DNA should thus subsequently have to be called R-DNA. However, T-DNA is the term commonly used, and this does not limit the invention to only *A. tumefaciens*, but includes all transformation methods in which a vector containing T-DNA is used.

For transformation, next to the T-DNA also virulence proteins are necessary. These can be coded for on the same plasmid which also contains the T-DNA, which in this constellation generally is referred to as a co-integrate plasmid. It can equally well be the case that the virulence genes are located on a separate plasmid (usually referred to as the binary vector system) or even on the bacterial chromosome.

Furthermore, the plasmids will normally also contain a gene coding for a resistance to an antibiotic (enabling culturing under selection pressure), and an *ori* for replication in *E. coli*.

A general introduction on the transformation of genes into plants and the role of *Agrobacterium* and the Ti-plasmid therein can be found in the handbook of "Principles of gene Manipulation" (Old, R.W. and Primrose, S.B., Blackwell Scientific Publications, London, 1994, Chapter 14, pp. 268-301).

One embodiment of the invention comprises a vector in which a gene coding for a toxin under the control of a plant-expressible promoter is located outside the T-DNA borders. It is essential that this gene is not toxic for or not expressed in the bacterium because that would deteriorate the bacterium and with it the capability of transformation of the T-DNA to the plant. There are several ways in which toxic effects for the bacterium can be prevented. One of them is to choose a toxic compound that is not toxic to the bacterium. An example of such a toxic compound is diphteria-toxin. Similarly, also the antisense approach in which an essential plant house-keeping gene is knocked out gives opportunities to prevent bacterial deterioration. For this it must be kept in mind to choose a house-keeping gene which is active (and essential) in plants, but which lacks, has only a minor function in bacteria or is not homologous enough to be hampered by antisense expression of the gene.

However, even if the toxins are harmful to bacteria, ways can be found to prevent expression of said toxins in the bacteria. One possibility to accomplish this is by producing a gene construct in which the toxin is under control of a plant promoter. Although not all plant-specific promoters can be used in such a vector, since they are give at least some expression in bacteria, it is easy to establish if a promoter of choice does not yield expression in bacterial cells (for instance by transforming a bacterium with a GUS-gene preceded by said promoter; expression of GUS in the bacteria can easily be assayed). Finally, a way to prevent expression in bacteria is to introduce an intron sequence into the coding sequence of the toxin (or to use intron-containing genomic sequences). Since bacteria are unable to excise introns only a non-functional (part of the) protein will be produced, which do not harm the bacteria.

Toxins which can be used comprise toxins specific for certain plants, but also the more general avaialable toxins which act on membrane systems and/or other general cell structures or processes. Examples of such toxins are: RIP, magainins, RNAses (like barnase), DNAses, proteases, etcetera.

Other approaches can be applied in several ways, and genes for these approaches may be selected from the group consisting of (a) genes encoding ribozymes against an endogenous RNA transcript, (b) genes which produce proteins which are able to evoke a hypersensitive reaction, (c) genes which when transcribed produce RNA transcripts that are complementary or at least partially complementary to RNA transcripts of endogenous genes that are essential for cell viability, a method known as antisense inhibition of gene expression (disclosed in EP-A 240 208), and (d) genes that when transcribed produce RNA transcripts that are identical or at least very similar to transcripts of endogenous genes that are essential for cell viability, an as yet unknown way of inhibition of gene expression referred to as co-suppression (disclosed by Napoli C. et al., 1990, The Plant Cell 2, 279-289).

Evoking a hypersensitive response (HR) is possible when a pathogen-derived elicitor protein and a corresponding plant-derived receptor protein are expressed simultaneously. Couples of such corresponding elicitor/receptor genes and their applicability to evoke a HR in a transgenic plant, are known in the art, e.g. for *Cladosporium fulvum* avr-genes and *Lycopersicon esculentum* Cf-genes (WO 91/15585) or for *Psuedomonas syringae* avr-genes and *Arabidopsis thaliana* RPM1-genes (Grant M.R., et al., Science 269, 843-846, 1995). The general idea of the application of these genes in this invention is to insert one of the genes between the T-DNA borders and the complementary gene outside the borders. Thus, when DNA from outside the borders is transferred to the plant both genes will be expressed and a hypersensitive response will be produced, which will kill the thus transformed cell.

Since the plant-derived resistance genes occur naturally in some of the plants it is possible for those plants to suffice with a gene coding for the corresponding avirulence gene situated outside the T-DNA borders. When expressed after transformation it will encounter the plant endogenously produced corresponding gene and evoke a HR response.

Preferred embodiments of the constructs for this HR-mechanism (in view of regulatory restrictions) are constructs in which the plant-derived gene is present between the T-DNA borders and the pathogen-derived gene is present outside the borders.

According to another embodiment of the invention use is made of antisense genes to inhibit expression of endogenous genes essential for cell viability, which genes are expressed in the plant cell.

Target genes for antisense disrupter genes are selected from those coding for enzymes that are essential for cell viability, also called housekeeping enzymes, and should be nuclear encoded, preferably as single copy genes, although a small size gene family would also be suitable for the purpose of the invention. Furthermore, the effect of antisense expression of said genes must not be nullified by diffusion or translocation from other cells or organelles of enzyme products normally synthesized by such enzymes. Preferably, genes coding for membrane-translocating enzymes are chosen as these are involved in establishing chemical gradients across organellar membranes.

Inhibition of such proteins by antisense expression can not, by definition, be cancelled by diffusion of substrates across the membrane in which these proteins reside. The translocated compound is not limited to organic molecules but can be of inorganic nature; e.g. P, H, OH or electrons.

A list of target enzymes is given in Table 1 by way of example but the invention is not limited to the enzymes mentioned in this table. More detailed listings can be assembled from series as Biochemistry of Plants (Eds. Stumpf & Conn, 1988-1991, Vols. 1-16 Academic Press) or Encyclopedia of Plant Physiology (New Series, 1976, Springer-Verlag, Berlin).

Although only in some cases, the genes coding for these enzymes have been isolated and, therefore, the number of gene copies are not known, the criteria that have to be met are described in this invention.

TABLE 1
EXAMPLES OF TARGET ENZYMES FOR ANTISENSE AND SENSE EXPRESSION

enzyme	pathway/organelle
ATP synthase	mitochondrion
adenine nucleotide translocator	mitochondrion
phosphate translocator	mitochondrion
tricarboxylate translocator	mitochondrion
dicarboxylate translocator	mitochondrion
2-oxo-glutarate translocator	mitochondrion
cytochrome C	mitochondrion
pyruvate kinase	glycolysis
glyceraldehyde-3P-dehydrogenase	glycolysis
NADPH-cytochrome P450 reductase	lipid metabolism
fatty acid synthase complex	lipid metabolism
glycerol-3P-acyltransferase	lipid metabolism
hydroxymethyl-glutaryl CoA reductase	mevalonic acid pathway
aminoacyl transferase	nucleic acid metabolism
transcription factors	nucleic acid metabolism
elongation factors	nucleic acid metabolism

To maximize the antisense effects in a plant host, the use of homologous genes is preferred. With homologous is meant obtainable from the same plant species as the plant host. Heterologous, for the purpose of this specication shall mean obtainable from a different plant or non-plant species. Heterologous shall also comprise synthetic analogs of genes, modified in their mRNA encoding nucleic acid sequence to diverge at least 5% of the host gene. As housekeeping genes are in general highly conserved, heterologous probes from other (plant) species can be used to isolate the corresponding gene from the crop species that is to be made resistant. Such gene isolations are well within reach of those skilled in the art and, in view of the present teaching require no undue experimentation.

As regards the necessity of a transcriptional terminator region, it is generally believed that such a region enhances the reliability as well as the efficiency of transcription in plant cells. Use thereof is therefore strongly preferred in the context of the present invention.

Another embodiment of the present invention is a vector in which read-through or starts of DNA-transfer from the left border are inhibited by insertion of a nucleotide sequence outside the T-DNA borders that interferes with the DNA unwinding process naturally needed for formation of a DNA molecule intended for translocation to the plant.
An example of such a sequence is a GC-rich sequence of approximately 40 nucleotides (preferable 20-60 basepairs), of which DNA unwinding is energetically unfavourable, and thus is expected to hamper unwinding of DNA through this sequence. However, also other sequences may be used which are blocking read-through or left-border starts. Calculations as to the increased stability of such double-stranded sequences are known to the person skilled in the art, and are described in Maniatis, Fritsch and Sambrook: Molecular Cloning, a laboratory manual, Cold Spring Harbor, 1982, pp 388.

Another example of a nucleotide sequence which will hamper the DNA unwinding process in sequences outside the T-DNA border is a sequence consisting of multimerised binding sites for *Agrobacterium* DNA-binding proteins. Displacement of bound double-stranded DNA-binding proteins will be required for unwinding of the DNA, so that the energy required for strand displacement is increased considerably.
In general, all double-stranded-DNA-binding proteins will be able to interfere with this process. Preferably, protein binding sites are used of which the DNA-protein interaction can be induced or strengthened by treatment of *Agrobacterium* cells with an external stimulus. More preferably, the DNA-binding protein is virG, which is also an activator protein for all vir proteins involved in T-DNA mobilization and transfer. VirG is known to bind to the vir box, consisting of the sequence 5'TNCAATTGAAAY3' (in which N is any nucleotide and Y is a pyrimidine base nucleotide (T or C)). Binding of virG to this vir box is thought to be initiated or augmented upon activation of the *Agrobacterium* through the virA/virG two-component regulatory system. *In vivo*, activation of vir genes is dependent on phosphorylation of virG, but the actual role of this modification is not yet known. As outlined in Sheng and Citovsky (The Plant Cell 8, 1699-1710, 1996) a very likely explanation is that phosphorylated virG protein has an increased affinity for its cognate binding site.

Also, introduction of binding sites for bound DNA-binding proteins closely linked to the left border sequences can physically interfere by steric hindrance with the assembly of proteins on this left border needed for strand displacement, thus effectively reducing starts at the left border.

Although some of the embodiments of the invention may not be practicable at present, *e.g.* because some plant species are as yet recalcitrant to genetic transformation, the practicing of the invention in such plant species is merely a matter of time and not a matter of principle, because the amenability to genetic transformation as such is of no relevance to the underlying embodiment of the invention.

Transformation of plant species is now routine for an impressive number of plant species, including both the *Dicotyledoneae* as well as the *Monocotyledoneae*. In principle any transformation method may be used to introduce chimeric DNA according to the invention into a suitable ancestor cell. A preferred method according to the invention comprises *Agrobacterium*-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP A 120 516 and U.S. Patent 4,940,838).

Tomato transformation is preferably done essentially as described by Van Roekel et al. (Van Roekel, J.S.C., Damm, B., Melchers, L.S., Hoekema, A. (1993). Factors influencing transformation frequency of tomato (*Lycopersicon esculentum*). Plant Cell Reports, 12, 644-647).

Potato transformation is preferably done essentially as described by Hoekema et al. (Hoekema, A., Huisman, M.J., Molendijk, L., van den Elzen, P.J.M., and Cornelissen, B.J.C. (1989). The genetic engineering of two commercial potato cultivars for resistance to potato virus X. Bio/Technology 7, 273-278).

Although considered somewhat more recalcitrant towards genetic transformation, monocotyledonous plants are amenable to transformation and fertile transgenic plants can be regenerated from transformed cells or embryos, or other plant material. Monocotyledonous plants, including commercially important crops such as rice and corn are amenable to DNA transfer by *Agrobacterium* strains (*vide* WO 94/00977; EP 0 159 418 B1; Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) Plant. Physiol. 95, 426-434).

It is known that practically all plants can be regenerated from cultured cells or tissues. The means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Shoots may be induced directly, or indirectly from callus via organogenesis or embryogenesis and subsequently rooted. Next to the selectable marker, the culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype and on the history of the culture. If these three variables

are controlled regeneration is usually reproducable and repeatable. After stable incorporation of the transformed gene sequences into the transgenic plants, the traits conferred by them can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

EXPERIMENTAL PART

EXAMPLE 1

Construction of a binary vector containing the Barnase gene outside the T-DNA region, downstream of the left border.

The binary vector pMOG800 contains a BglII site outside the T-DNA, close to the left border (about 450 bp downstream the left border sequence of the T-DNA). This site is used to introduce a DNA fragment containing a GAPc-promoter, Barnase encoding DNA including an intron to prevent expression of the barnase gene in a bacterial host, and a nos-terminator. The latter construct is prepared as followed: the GAPc-promoter is obtained as a BamHI-NcoI DNA fragment (812 bp), isolated from pMOG950 (originally denominated pBZ101, kindly provided by Ming-Che Shih, department of Biological Sciences, Iowa, USA). A barnase/intron, nos-terminator containing DNA fragment is obtained by digestion of the binary vector pMOG944 with NcoI and EcoRI as a 629 bp fragment. The two fragments will be ligated together (3-points ligation) in the high-copy vector pBSK digested with BamHI and EcoRI. Clones containing the expected construct, as determined by restriction of the DNA and subsequent DNA sequencing of the apparently correct clones, can now be digested with BamHI, which will give a 1.4 kb fragment containing GAPc-Barnase/Intron-TPI-II. The fragment is cloned into this BglII site of pMOG800 and pMOG1059. The latter is the pMOG800-derived binary vector containing the GUS-intron reporter gene behind a strong constitutive promoter and is denominated 'binary selector'.

EXAMPLE 2

Testing transformation efficiency and functionality of the binary selector vector containing a barnase/intron expression cassette.

In order to test the efficiency of stable integration events of the new binary selector vector in plants, the GAP-C barnase/intron expression unit is cloned outside the T-DNA region of pMOG1059 containing a GUS/intron expression cassette cloned in the T-DNA upstream from the selectable marker. The resulting 'binary selector' vector denominated pMOG1256 (fig. 1), and the parental pMOG1059 vector are transformed to *Arabidopsis thaliana* plants by using infection with *Agrobacterium tumefaciens* strains harbouring these binary vectors. Callus is screened for GUS-activity, indicative for stable integration of the T-DNA.

Shoots of transformed plant tissue are tested for the presence of outer-border sequences by PCR. PCR reactions are carried out using isolated genomic DNA of the transformants: (1) a PCR reaction of which the product encompasses the left-border, for detection of left border read-through, using primers 5'-CAT CGG TAA CAT GAG CAA AGT CTG-3', and 5'-GAC GCT AAA GGC AAA CTT GAT TC-3', (2) a PCR reaction of which the product encompasses the right-border sequence to detect right-border read-through, carried out using primers 5'-GAG ATC AGA TTG TCG TTT CCC GCC TTC-3' and 5'-CCA ACT TAT CAG TGA TAA AGA ATC CGC-3'.

EXAMPLE 3

Determination of frequencies of stably integrated outside T-DNA vector sequences of the binary vector in different plant species.

To access the frequency of stable integration events of binary vector outer-border sequences in different plant species a constitutive promoter + GUSintron + TPI+II terminator reporter unit from pMOG1059 is introduced in the BglII site in the left border-flanking sequence of pMOG800, outside the T-DNA borders. After transformation of *Arabidopsis*, tobacco, potato, tomato and oilseed rape, using

Agrobacterium tumefaciens, regenerated callus tissue is screened for GUS activity, which shows the presence of vector sequence insertion, of which the sequence contains at least a functional GUS-reporter unit. As a control pMOG1059 is transformed side-by-side to determine the efficiency of stable T-DNA sequences integration.

EXAMPLE 4

Introduction of a GC-clamp in the binary vector outer-border sequence close to the left-border.

To decrease the frequency at which T-DNA outer-border sequences are mobilized and transfered to the plant, a GC-clamp is introduced outside the T-DNA within a distance of 10 bp to the left-border 24 bp recognition sequence. A GC-rich stretch of 40 nucleotides with a GC content of 90% is introduced as two complementary oligonucleotides which are annealed, so that double stranded DNA fragments are created. By means of their ends that form compatible overhangs, these DNA fragments are ligated into multimerized forms and subsequently cloned into a restriction site that is introduced just outside the T-DNA flanking the left T-DNA border. Like the experiments described above a constitutive promoter-GUS cassette is present in the vector sequences outside the left T-DNA border.
The efficacy of the new construct in decreasing the introduction of non T-DNA vector sequences in transgenics is checked by introducing a GUS reporter unit in the BglII site and determine GUS activity in callus tissue as described above.

EXAMPLE 5

Introduction of multiple vir box sites outside the left T-DNA border

Two complementary oligonucleotides containing the sequence of vir boxes are annealed, so that double stranded DNA fragments are created. By means of their ends that form compatible overhangs, these DNA fragments are ligated into multimerized forms and subsequently cloned into a restriction site that is introduced just outside the T-DNA

flanking the left T-DNA border. Like the experiments described above a constitutive promoter-GUS cassette is present in the vector sequences outside the left T-DNA border.

The resulting binary vector and its corresponding parental vector, lacking the multimerised vir boxes is introduced into Agrobacterium tumefaciens and used in transformation experiments in Arabidopsis thaliana, tomato, potato and Brassica napus.

Transformed plants are analysed for presence of non T-DNA vector DNA by GUS staining.

EXAMPLE 6

Use of avirulence and resistance genes for counterselection of vector sequences outside the T-DNA.

The *Pseudomonas syringae*-derived AvrRpm1 elicitor coding region is cloned operatively behind a strong constitutive promoter, and before a the potato proteinase inhibitor II transcriptional terminator sequence. This cassette is introduced into the BglII site downstream of the left T-DNA border. A genomic DNA fragment containing the *Arabidopsis thaliana* derived Rpm1 resistance gene flanked by a constitutive promoter and terminator sequences is introduced into the T-DNA, thus forming pMOG 1257 (fig. 2). Transformation of Brassica napus, tomato and potato plants with this construct is performed using standard procedures.

Transgenic plants arising from this transformation procedure are analysed for their presence of vector sequences outside the T-DNA by using a PCR reaction on genomic DNA of each of the transformants. The primers used span a sequence of appr. 300 bp outside the T-DNA close to the left border.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MOGEN International nv
(B) STREET: Einsteinweg 97
(C) CITY: LEIDEN
(E) COUNTRY: The Netherlands
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(ii) TITLE OF INVENTION: Novel plasmids for plant transformation and method for using same.

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAGATCAGAT TGTCGTTTCC CGCCTTC 27

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCAACTTATC AGTGATAAAG AATCCGC 27

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CATCGGTAAC ATGAGCAAAG TCTG 24

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GACGCTAAAG GCAAACTTGA TTC 23

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TNCAATTGAA AY 12

CLAIMS

1. Vector for plant transformation comprising a T-DNA with flanking T-DNA borders, characterized in that the vector further comprises a nucleic acid sequence which prevents the development of plant transformants having more vector sequences than the T-DNA sequence.

2. Vector according to claim 1, characterized in that the nucleic acid sequence which prevents the development of transformants having more vector sequences than the T-DNA sequence is a gene coding for a toxic compound, preferably selected from the group of RNAse, DNAse, phytotoxins, diphteria toxin, proteases and antisense housekeeping genes, such as ATP synthase, cytochrome c, pyruvate kinase, aminoacyl transferase, or phosphate, di-, tricarboxylkate and 2-oxo-glutarate translocators.

3. Vector according to claim 1, characterized in that the nucleic acid sequence which prevents the development of transformants having more vector sequences than the T-DNA sequence does not allow readthrough by comprising a sequence which prohibits unwinding of the DNA.

4. Vector according to claim 3, characterized in that the nucleic acid sequence prevents the development of transformants having more vector sequences than the T-DNA sequence by comprising a sequence which binds DNA-binding proteins.

5. Vector according to claim 4, characterized in that the sequence which binds DNA-binding proteins is a vir box, preferably the sequence 5'TNCAATTGAAAY3' (in which N is any nucleotide and Y is a pyrimidine base nucleotide (T or C)).

6. Vector according to claim 5, characterized in that the sequence which prohibits unwinding of the DNA is a sequence which has a high GC-content, preferably a sequence of 20-60 basepairs, more preferably a sequence of about 40 basepairs.

7. Vector according to claim 5 or 6, characterized in that the sequence has a GC-content of more than 80%, preferably more than 90%.

8. Method for obtaining transgenic plants which do not contain vector sequences outside the T-DNA by transforming plants with a vector according to any of claims 1-7.

9. Host containing a vector according to any of claims 1-7.

10. Host of claim 5, characterized in that it is a bacterium, preferably a memeber of the Agrobacteriaceae, more preferably *Agrobacterium* or *Rhizobacterium*, most preferably *Agrobacterium tumefaciens*.

11. Method for the transformation of plants characterized in that a vector of any of claims 1 to 7 is used.

ABSTRACT

This invention relates to the *Agrobacterium* mediated plant transformation, especially to transformation of plants with T-DNA, where read-through at the borders is prohibited. This can be done by inhibition of transfer of vector DNA to the plant cell by creating DNA binding sites outside the T-borders, but also to insert a coding sequence outside the T-borders which, whether or not in cooperation with genes from the host plant or genes cotransferred, are toxic to plants so that there is counterselection for plants with superfluous vector-DNA.

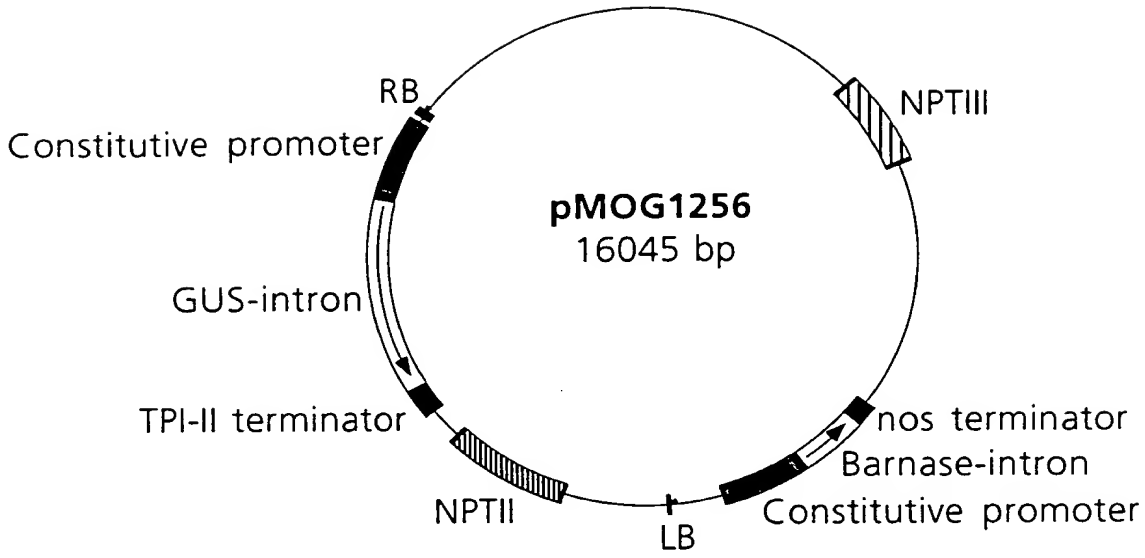
RB
NPTIII
Constitutive promoter
pMOG1256
16045 bp
GUS-intron
TPI-II terminator
nos terminator
Barnase-intron
NPTII
LB
Constitutive promoter

Fig. 1

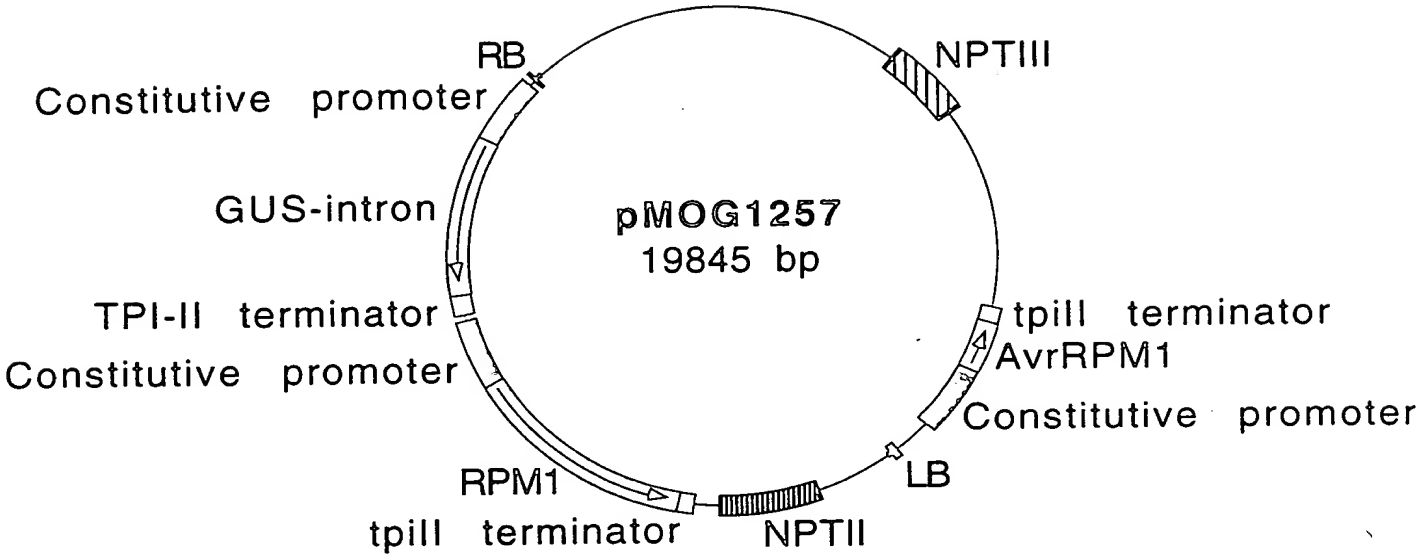
RB
NPTIII
Constitutive promoter
GUS-intron
pMOG1257
19845 bp
TPI-II terminator
tpiII terminator
Constitutive promoter
AvrRPM1
Constitutive promoter
LB
RPM1
tpiII terminator
NPTII

Fig. 2